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(54) Title: ALLERGEN PEPTIDES

(57) Abstract

Synthetic peptides have an amino acid sequence which includes at least one human antigenic determinant of a Kentucky Blue grass (KBG) allergen from the Poa p IX group of grass pollen allergens, specifically the KBG 60 allergen.

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TITLE OF INVENTION
ALLERGEN PEPTIDES

FIELD OF INVENTION

The present invention relates to the provision of synthetic peptides which contain epitopes of a specific class of Kentucky Blue grass allergen.

BACKGROUND TO THE INVENTION

Pollen allergens are multideterminant proteins or glycoproteins capable of eliciting IgE-mediated allergic diseases such as hayfever and asthma in approximately 17% of the population who are genetically predisposed to develop allergies (ref. 1 - a list of the references appears at the end of the disclosure). In contrast to some other allergens (e.g., cat or house dust mite allergens), the global distribution of pollens of a large variety of monocotyledonous (grasses) and dicotyledonous plants (trees and weeds) preclude any realistic possibilities that individuals allergic to pollen allergens can avoid these aero-allergens.

Upon initial exposure to allergenic constituents, they are internalized by antigen-presenting cells (APC), which include nonantigen-specific phagocytic cells or specific B cells, and are "processed" by these cells. The net effect of this processing is the breakdown of the antigens into peptidic determinants which, in turn, are re-expressed in association with class I or class II molecules of the major histocompatibility complex (MHC) on the surface of the APC. Subsequently, the binary peptide-MHC complexes interact with the corresponding specific receptors (TCR) of, respectively, (i) Th cells or (ii) CTLs or Ts cells, and the resulting triads determine the up- or down-regulation of the appropriate B cells (ref. 13).

On the basis of their lymphokine secretion patterns, the Th cell subpopulation may be further subdivided into three subsets, i.e., Th0, Th1 and Th2 cells (ref. 14).

In mice and man, the Th2 cells have been shown to produce IL-4, IL-5 and IL-6, and IL-4 has been shown to activate B cells leading to the production of IgE antibodies. In contrast, the Th1 cells produce IFNγ, which blocks the production of IL-4. The mechanism(s) involved in the suppression of antibody responses by Ts cells is still not fully understood. It has been suggested that the suppression of antibody production is due to inactivation of specific Th cells as a result of non-professional APC, i.e. a T-cell (ref. 15).

The primary reaction of the IgE antibodies secreted from the Bε cells is their binding to specific IgE receptors on the surface of mast cells, basophils and eosinophils. On re-exposure of the patient to the specific multivalent allergen, the cell-fixed IgE antibodies react with and are crosslinked by th allergenic molecules. This process leads to the release from these cells of chemical mediators of anaphylaxis. In turn, these mediators act rapidly on the smooth muscles of different target organs, resulting in the inflammatory manifestations characteristic of immediate type hypersensitivity.

The development of therapeutic strategies (ref. 16) that may influence the formation of IgE antibodies requires detailed knowledge of (i) the structures of individual allergens and, in particular, of their B cell epitopes (which are recognized by IgE/IgG antibodies), (ii) structures recognized by MHC molecule (i.e., Ia epitope), and (iii) the structures recognized by T-cell receptors of Th or Ts cells, known as T-cell epitopes. Therefore, determination of primary sequences of individual pollen allergens of a complex repertoire of allergens of a given pollen by immunochemical and physiochemical methods is of central importance, and has been a major obj ctive in allergy research for a long time.

The current treatment for hayfever consists primarily in symptomatic relief. Sufferers take drugs, such as anti-histamines and steroids, which do not suppress the formation of IgE antibodies and often have harmful side effects.

Attempts to downregulate the IgE immune responses of allergic subjects by the "time-honoured" immunotherapy consist of a series of injections of increasing amounts of the allergenic extracts of the appropriate pollen or pollen-mixtures over prolonged periods lasting usually 3 to 5 years. Most of the pollen extracts used therapeutically are crude mixtures of a multiplicity of chemical constituents, some of these components bearing no relation whatsoever to the few allergenic components which are actually responsible for a given patient's hypersensitivity. Because some of the proteins present in these extracts may not be allergens, standardization of allergenic extracts based on total protein content is an unreliable guide for determining the potency of an extract. Moreover, large (up to 100x) variations in allergen content occur in the preparations used for immunotherapy because of the different methods used for (i) pollen collection and storage, which lead to variations in raw materials from lot to lot and from year to year, and (ii) the extraction procedures. Moreover, although different patients may be allergic to different constituents of a given pollen, all patients receive injections of the "same" complex mixture containing all the constituents of different pollens, i.e., they receiv even components to which they may not be allergic. It is, therefore, not surprising that treatment with an ill-defined pollen extract may lead to the induction of additional IgE antibodies, i.e., to sensitization of the patients to new components (refs. 2 to 6).

While up to 80% of patients gain clinical improvement from this immunotherapy (refs. 7, 8), the

risk of side effects, the lengthy course of therapy, the inconvenience to the patient of the mode and frequency of administration, and the mounting costs of this treatment limit the utility of the current immunotherapy. Although, local and systemic reactions may occur as a result of this therapy, they may be managed by a physician specialized in allergy. However, occasionally this mode of treatment is associated with the risk of severe asthmatic or anaphylactic reactions, which can result in death (refs. 9, 10).

To eliminate some of the above disadvantages of the allergenic preparations currently used for immunotherapy, one of the major objectives in allergy research has been the isolation and characterization of the individual allergens of the complex repertoire of allergens of a given pollen by physicochemical and immunochemical methods. Several laboratories have isolated some of the allergens from the crude aqueous extracts of grass pollens by the use of classical physiochemical methods and reverse immunosorbents consisting of immobilized murine monoclonal antibodies to the pollen constituents (refs. 11, 12). Although such immunochemical methods appear to be promising for the characterization of individual allergens, the main drawback of these extremely labour intensive purification methods is the minute yield of allergens. Moreover, these methods do not ensure absolute purity of the allergenic constituents and, therefore, the determination of their amino acid sequences is difficult, if not impossible. As a corollary, the development of new therapeutic derivatives of grass pollen allergens and of reliable diagnostic procedures for pollen allergies are severely restricted by the use of allergens isolated by the existing procedures. However, recent innovations in recombinant DNA (rDNA) technology have paved the way for the synthesis of allergenic proteins, and of their epitopic

fragments responsible for their activation of the appropriate B- and T-cells leading interactively to IgE formation, on an industrial scale and in a consistently pure state.

However, using these classical methods, the progress in isolation and characterization of allergens has been slow. For example, for ragweed pollen, which is one of the main allergenic pollens in North America, in spite of intensive studies of over 50 years only six allergens (i.e., Amb a I, Amb a II, Amb a III, Amb a V, Amb a VI and Amb t V) have been purified and characterized (refs. 17 to 19). Similarly among grass pollens, one may cite pollens of Ryegrass, Timothy grass and Kentucky Blue grass, and many others, which have been used for the isolation and characterization of their protein allergens (refs. 20 to 25).

Similar to other protein antigens, the epitopes of allergenic proteins are either continuous or discontinuous. Generally, continuous antigenic epitopes can be localized to segments composed of amino acid residues in a linear sequence, whereas discontinuous (conformational) epitopes comprise residues which appear adjacent to one another on the protein surface, but ar widely separated in their primary sequence. The latter epitopes depend on the native conformation of th protein. Conventional methods for identification of B cell epitopes consist of probing a polyspecific antiserum or a set of monoclonal antibodies produced against the intact antigen with cleavage fragments of antigen or synthetic peptides, which may yield information on continuous epitopes (refs. 26 to 27). Thus, on the basis of amino acid sequence data for Amb a III, Atassi and his associates synthesized ten overlapping pentadecapeptides which represent d the entire Amb a III molecule (ref. 28). These peptides served to localiz four antigenic sites in Amb a III that were recognized by IgG antibodies

in human, rabbit and murine antisera. It is noted that the same regions were also recognized by human IgE antibodies (ref. 29).

Furthermore, by coupling partially-purified ragweed pollen allergens to monomethoxypolyethylene glycol (mPEG), it was shown that the resulting conjugates were (i) not only devoid of allergenicity and immunogenicity of the original ragweed pollen constituents in patients and mice, respectively, (ii) but were also capable of inducing a long-lasting suppression of IgE antibodies in mice (ref. 30). It was also reported that conjugates of poly-(N-vinyl pyrrolidone) with Timothy grass pollen allergens suppressed the established IgE responses to these allergens in mice (ref. 30).

Because of the limitation of the above classical purification methods which yield only minute amounts of pure allergens, recently some investigators have used the rDNA methods for the study of allergens. Thus, allergens present in dust mite (refs. 31 to 33), hornet venom (ref. 34), birch pollen (ref. 35) and grass pollens (ref. 36) have been cloned and the respective allergens produced by the application of rDNA techniques.

On the basis of sequence homology and cross-reactivities, the grass allergens cloned to-date may be classified to two main groups, one group includes allergens of 11 and 35 kDa in size (ref. 13) and th other group includes allergens of 28 to 34 kDa in size. Published Canadian patent application No. 2,068,694, in which we are named as coinventors (corresponding to copending United States patent application Serial No. 206,723 filed March 7, 1994, the disclosures of which are incorporated herein by reference) discloses the cloning of the cDNAs of three isoallergens of Kentucky Blue Grass Poa pratensis (KBG) pollen and identified immunologically important regions within these latter group of allergens. These allergens are present in some other grasses and are

thus useful for diagnosis of and desensitizing for grass-specific allergies.

The latter document describes a procedure for defining the antibody binding sites of the grass allergen rKBG60 using overlapping cDNA clones and peptides synthesized on polypropylin pins with the aid of murine antisera to rKBG60. The authors failed to detect IgE-binding epitopes using the decapeptides on the pins.

The development of therapeutic strategies for allergic diseases that would specifically regulate IgE antibody production to defined allergens requires knowledge of the primary structure of individual allergens, in particular, the knowledge of their epitopes, which are recognized by antibodies and T-cells (ref. 37). Thus, the peptides comprising the B cell epitopes may be exploited for devising the epitope-specific diagnosis (ref. 16) and for inducing allergen-specific B cell tolerance (ref. 38). Similarly, the T-cell epitopes are considered relevant since the structural analogues of the corresponding peptides may be used to manipulate T-cell responses (refs. 39 to 42).

The B cell epitopes may comprise IgG-binding epitopes (ref. 43) and/or the IgE-binding epitopes of allergens, and may be also sequential or conformational in nature (refs. 44 to 48). Evidence gathered so far from epitope mapping of a few allergens indicate that IgG and IgE antibodies recognize the same or similar epitopes on allergens (refs. 28, 49 to 52). Expression of various antibody isotypes have been reported to be controlled by lymphokines secreted by different subsets of T-cells (refs. 53 to 55). In contrast to the B cell epitopes, the epitopes recognized by T-cells are linear in nature. T-cell epitopes of allergens are pivotal to the T-cell activation and may also play a role in differentiation of these T-cell subsets (refs. 39 to 41). Moreover, recent studies suggest that T-cell epitopes may be exploited for

induction of allergen-specific tolerance in vivo (ref. 56). Definition of T-cell epitopes, therefore, is not only required for a further understanding of their roles in T-cell differentiation, but also essential for designing more effective immunotherapeutic strategies.

SUMMARY OF INVENTION

The present invention provides synthetic peptides which contain human T-cell and/or B-cell epitopes of a Kentucky Blue grass (KBG) allergen from the Poa p group of grass pollen allergens, in particular the rKBG60 from Kentucky Blue Grass pollen described in aforementioned CA 2,068,694 and USSN 206,723. In particular, the analysis of B- and T-cell epitopes of rKBG60 described therein has been extended utilizing 20-residue long synthetic peptides each with a 10-residue overlap, for further defining the T- and B-cell epitopes in humans and mice.

In accordance with the present invention, there is provided a synthetic peptide having an amino acid sequence which includes at least one human antigenic determinant of a Kentucky Blue Grass (KBG) allergen from the Poa p IX group of grass pollen allergens, particularly the KBG allergen KBG 60 but including any other KBG allergen from this group. The human antigenic determinant may comprise a human T-cell epitope, a human B-cell epitope or both. The synthetic peptide may have an amino acid sequence corresponding to one of the sequences shown in Table 1 below which exhibit a human B-cell and/or a human T-cell response.

In particular, the synthetic peptide may be selected from peptides 6, 9, 10, 11, 12, 17, 21, 23 and 28 in Table 1, exhibiting IgG human B-cell response, peptides 12, 13, 18, 20, 21, 23, 24, 25, 26, 27 and 28 in Table 1, exhibiting IgG human B-cell response, and/or peptides 6, 9, 10, 13, 14, 17, 18, 19, 20, 21, 23, 25, 26, 27 and 28 in Table 1, exhibiting human T-cell responses. Specific

peptide may comprise peptides 13, 14, 25 or 26 in Table 1.

The present invention additionally provides an antiserum specific for a synthetic peptide as provided herein.

The present invention further includes a composition for protecting an allergic individual from developing an allergic reaction to grass pollen, comprising at l ast one synthetic peptide as provided herein and a pharmaceutically-acceptable carrier therefor. Such composition may be formulated as a vaccine for in vivo administration.

The vaccine may comprise the at least one synthetic peptide conjugated to a non-immunogenic substrate, particularly a polymeric material, which may be a carboxymethycellulose, monomethoxypolyethylene glycol or polyvinyl alcohol. The non-immunogenic substrate also may comprise beads for targeted uptake of at least one recombinant protein by selected antigen-preventing cells.

The composition comprising the synthetic peptide may be formulated as a micropeptide, capsule or liposome preparation and may be provided in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces. The vaccine may further comprise at least one additional desensitizing agent, which may be selected from the group consisting of Lol p I allergen, Bet v I allergen, Amb a I allergen, Amb a II allergen and CRAL 51 allergen.

The vaccine composition may further comprise at least one compound having anti-histamine activity and/or at least one compound having anti-inflammatory activity and/or at least one compound which is immunosuppressive. The vaccine composition may further comprise an adjuvant.

The present invention further comprises a method of desensitizing an allergic individual, particularly a human, by administering to the individual an effective

amount of the peptide-containing composition provided herein.

In an additional aspect, the present invention provides a method of depleting allergen-specific antibodies from an individual, comprising contacting the antibodies with the peptide-containing composition provided herein to form a complex, and removing the complex from the individual, particularly a human.

A further aspect of the present invention provides a method of anergizing allergen-specific antibody-producing cells, which comprises contacting the cells with a synthetic peptide-containing composition as provided herein.

The present invention additionally provides a method for diagnosing an allergic reaction to grass pollens, which comprises administering to an individual, particularly a human, a synthetic peptide as provided herein, and evaluating a response to with administration. A further diagnostic procedure provided herein comprises contacting serum from an individual, particularly a human, with a synthetic peptide as provided herein, and determining the formation of a complex between the synthetic peptide and pollen-specific IgE antibodies present in the serum.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows determination of human IgG-binding epitopes on rKBG60 allergen. The reactivity of the overlapping synthetic peptides with the IgG Ab of the serum pools of the KBG-allergic individuals (a) and non-KBG allergic individuals (b) was determined by ELISA;

Figure 2 shows the determination of human IgE-binding epitopes on rKBG60 allergen. The IgE-binding epitopes were identified by the reactivity of overlapping synthetic peptides with IgE antibodies in serum pools of the KBG-allergic individuals (a) and non-KBG allergic individuals (b) using ELISA assay;

Figure 3 shows the analysis of murine antibody-binding epitopes on rKBG60 allergen. The murine antibody-reactive peptides were defined by the reactivity of the synthetic peptides with Abs in anti-rKBG60 (a) and pre-immune (negative control) sera (b) by ELISA;

Figure 4 shows the proliferative responses of PBMCs for eight specific individuals (identified by initials). 5 x 10^5 PBMCs were cultured in the presence of different peptides at 100 µg/ml for 5 days. The values given are the arithmetric means cpm of [^{3}H]-thymidine incorporation in triplicate cultures and the cpm to peptides with background subtracted are shown;

Figure 5 shows the induction of allergen-specific antibodies by the synthetic peptides. The antisera from the mice immunized with the peptides were assayed by ELISA to determine their bindings to the rKBG8.3. Th non-immunized mouse sera (NS) were used as negative control;

Figure 6 shows the reactivities of the synthetic peptides with the antibodies induced by the same or other peptides. Each of the peptides used for immunization was coated onto the ELISA plate to determine its ability to bind to the anti-peptide sera. Two sets of experiments were performed with six peptides used as coating antig ns in each set; Set A included peptides #5, #18, #19, #21, #22 and #23 and Set B included peptides #14, #16, #25, #26, #27 and #28, as identified in Table 1 (below);

Figure 7 contains the cDNA sequences (SEQ ID NOS: 1 to 3) of KBG clones 41, 60 and 31. Sequences are broken into codons, have gaps inserted, and are aligned within the translated region to show maximum similarly. Dots represents homology with clone KBG41. Underlined regions are polyadenylation signals. Double underlined regions on clone 41 indicate internal repeat. Lowercase characters are EcoRI linkers. Numerical values at the start of each line refer to clone number and the number

of the nucleotide residues from the A in the start codon; and

Figure 8 contains the translated amino acid sequences (SEQ ID NOS: 4, 5 and 6) of KBG clones 41, 60 and 31. Sequences are aligned and have gaps aligned to show maximum similarly between clones. The alignments are the same as those in Figure 7. Putative signal peptides are in lowercase characters. Underlined regions in KBG31 are predicted B-cell epitopes. Double underlined region on clone 41 shows internal repeat. Numerical values at the start of each line refer to the clone and the number of amino acid residues from putative cleavage site of the signal peptide.

GENERAL DESCRIPTION OF INVENTION

The work described herein, aimed at further analysis of the epitopes of the rKBG60 allergen using synthetic 20-residue peptides having the amino acid sequences shown in Table 1 (below) (SEQ ID NOS: 7 to 26), has enabled us (i) to localize human IgG- and human IgE-binding epitopes, (ii) to identify the peptides capable of stimulating human T-cells in PBMCs, (iii) to confirm the B cell epitopes previously deduced from the study of antibody-binding peptides by the pin method, as described in CA 2,068,694 and USSN 206,723, and (iv) to determine if the peptides recognizing B and T-cells are also capable of eliciting antibodies in mice.

The work described here shows that the human IgE epitopes on the rKBG60 are localized predominantly on the C-terminal region of the molecule. In contrast to the human IgE epitopes, human IgG and murine antibody binding epitopes have been found to be localized on the c ntral region on the primary structure of the rKBG60 (see Table 1). The mapping of murine antibody-binding epitopes with these synthetic overlapping peptides was consistent with the results of binding of the recombinant polypeptid s with murine antibodies, as previously described in CA

2,068,694 and USSN 206,723. The comparison of the peptides recognized by human IgG and IgE and murine antibodies showed that most of the human IgE binding peptides were able to react with human IgG and murine antibodies. Moreover, a few peptides recognized only by human IgE antibodies were always adjacent to the epitopes of murine or human IgG antibodies.

The reason for the differences between epitopes recognized by human and murine antibodies currently is unknown. This difference may be due to the differences in the mode, frequency and/or route of immunization between mice and humans and the MHC differences. Similarly, at present the basis for this differential binding of human IgG and IgE binding of some peptides is unknown. Since the V gene pool for both isotype of antibodies is same, this difference may reflect the different concentration of antibodies present in this pool of sera of chronic allergic patients.

One of the approaches to detect the T-cell epitopes taken herein comprised the proliferation of PBMC of allergic humans in response to overlapping peptides. The results demonstrated that rKBG60 allergen contained a number of T-cell epitopes which varied with each individual, nevertheless certain epitopes were recognized more frequently by T-cells of different individuals than others. These results are consistent with recent studies with tree allergens (ref. 57). Moreover, the peptides differed in terms of their ability to proliferate PBMCs.

An additional approach for determining the T-cell epitopes was by examining the immunogencity of peptides in mice. The latter approach is based on the principl that a short peptide can elicit antibody production only if it possesses three sites, i.e., sites recognized by B cell surface Ig, class II MHC molecule, and T-cell receptor (refs. 57 to 60). Accordingly, the antibody-binding peptides which elicited allergen-specific

antibodies without coupling to a carrier macromolecule, are considered to possess T-cell epitopes. The results obtained demonstrated that the T-cell epitopes determined by these two different methods led to similar conclusions, i.e., the T-cell epitopes were localized on the same synthetic peptide, suggesting that the human and murine T-cell epitopes may be similar. These results were in agreement with Atassi et al (ref. 28). The fact that the same peptides also bound to antibodies indicat d that the T- and B-cell epitopes are present at close proximity to each other.

T-cell epitopes from foreign proteins provide help to the B-cell epitopes to induce antibodies, which recognize the corresponding native proteins, when the two kinds of epitopes are linked (refs. 61, 62). Thus, eight out of twelve synthetic peptides used in this study, induced antibodies which not only recognized the peptides used as immunogen but also the intact antigen which is consistent with previous studies (ref. 63). However, the antibodies induced by each of the peptides #5, #18, #22 and #28 bound weakly or not at all with the corresponding peptide but reacted with an adjacent overlapping peptide. It was therefore, considered necessary to use adjacent peptides to confirm their immunogenicity. Furthermore, the antibodies induced by the peptides #25 to 28 w re cross-reactive with each other, suggesting the presence of common antibody-binding epitope which may b conformational in nature.

The potential of immunotherapy with T-cell epitopes is being considered by some laboratories, in particular using the peptides of Fel d I, which constitut s the major allergen of the cat dander (ref. 56). Since this allergen contains only four T-cell epitopes, it is likely that a combination of T-cell epitopes may substitute th crude extract for therapeutic purpos s. However, th situation is more complex with the grass pollens, which

comprise at least three major groups of allergens. These studies with rKBG60 described herein, one of these grass allergens, show that the epitope recognition profiles by grass-allergic individuals is more diverse and, therefore, a combination of epitopes may be difficult, if not impossible. Furthermore, in this grass allergen the antibody binding residues flank the residues recognizing T-cells and thus, therapeutic peptides have to b thoroughly curtailed to avoid IgE binding ability of these peptides and consequently the possibility of systemic reactions. For example, although three predominant T-cell reactive regions were identified on rKBG60, the peptides #18 to 19 and #25 to 28 also bind to IgE antibodies, only peptide #13 to 14 may be readily utilized for T-cell epitope-based therapy.

While there is described herein the specific identification of amino acid sequences containing B- and/or T-cell epitopes of the rKBG60 allergen, the considerable sequence homology among the KBG clones 41, 60 and 31 (see Figures 7 and 8) indicates that corresponding epitope-containing amino acid sequences ar provided by the corresponding sections of the rKBG41 and rKBG31 allergen. As described in the aforementioned CA 2,068,694 and corresponding USSN 206,723, such allergen comprises a new group of grass pollen isoallergens, designated Poa p IX. The present invention includes amino acid sequences containing at least one antigenic determinant of any Kentucky Blue grass allergen from the Poa p IX group of grass pollen allergens.

Since the rKBG60-group of allergens comprise a major group of allergens in grass pollens, the work described herein provides a framework of important considerat ons in relation to immunotherapy with peptides for grass pollen allergies.

Compositions, suitable to be used for protecting allergic individuals from developing an allergic

reaction, may be prepared from the Kentucky Blue grass allergen peptides disclosed herein. Compositions may be prepared as injectables, as liquid solutions or emulsions. The Poa p IX allergen peptides may be mixed with pharmaceutically-acceptable excipients which are compatible with the allergen proteins, fragment analogs or peptides. Excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The composition may further contain minor amounts of auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Methods of achieving an adjuvant effect for the compositions includes the use of agents, such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline. Compositions may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10 to 95% of the allergen peptides.

The compositions are administered in a manner compatible with the dosage formulation, and in such amount as is therapeutically effective to protect allergic individuals from developing an allergic reaction. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies. Precise amounts and identity allergen

peptide required to be administered depends on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of nanograms to micrograms of the allergen peptides. Suitable regimens for initial administration and booster does are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the composition may also depend on the route of administration and will vary according to the size of th host.

The allergen peptides also may be conjugated to a non-immunogenic substrate including polymeric materials, such as carboxymethyl celluloses, monomethoxypolyethylene glycols (mPEG) and polyvinyl alcohols to render it non-immunogenic and non-allergenic for protecting allergic individuals from developing an allergic reaction. mPEG conjugates to a variety of allergens have been tested in human clinical trials (ref. 45).

The use of the allergen peptides provided herein <u>in vivo</u> may first require their chemical modification, since the peptides themselves may not have a sufficiently long serum and/or tissue half-life. Such chemically modified peptides are referred to herein as a "peptoid". The term "peptoid" extends to any functional chemical equivalent of a peptide characterized by its increased stability and/or efficacy <u>in vivo</u> or <u>in vitro</u> in respect of the practice of the invention. The term "peptoid" is also used herein to extend to any amino acid derivative of the peptides as described herein. Peptide analogs contemplated herein are produced by procedures that include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptid synthesis and the use of cross-linkers and other methods which impose

conformational constraint on the peptides or their analogs.

Examples of side chain modifications contemplated by the present invention include modification of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acetylation with acetic anhydride; carbamylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2,4,6, trinitrobenzene sulfonic acid (TNBS); alkylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxa-5'-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents, such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation via o-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulfhydryl groups may be modified by methods, such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of mixed disulphides with other thiol compounds; reaction with maleimide; maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulfonic acid, phenylmercury chlorid , 2-chloromercuric-4-nitrophenol and other mercurials; carbamylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tryosine residues may be altered by

nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid-, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

The allergen peptides described herein are advantageous as diagnostic reagents and antigens for the production of allergen-specific antisera.

The allergen peptides of the present invention are useful as immunogens, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of allergen specific IgE antibodies. In ELISA assays the allergen peptides are immobilized onto a selected surface, for example, a surface exhibiting a protein affinity, such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed allergen peptide, a nonspecific protein, such as bovine serum albumin (BSA) or casein that is known to be antigenically neutral with regard to the test sample, may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface. Normally, the peptides provided herein ar at least 12 residues in length and preferably 14 to 30 residues. It is understood however, that a mixture of

peptides may be used either as an immunogen in a composition or as a diagnostic agent.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures, such as of the order of 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplex s between the test sample and the bound allergen peptides, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody would be an antibody having specificity for human IgE or IgG antibodies. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity that will generate, for example, a color development upon incubating with an appropriate chromogenic substrate. Quantification may then achieved by measuring the degree of color generation using, for example, a visible spectra spectrophotometer.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of th invention. Although specific terms have been

employed herein, such terms are intended in a descriptiv sense and not for purposes of limitations. Immunological and recombinant DNA methods may not be explicitly described in this disclosure but are well within the scope of those skilled in the art.

EXAMPLES

Example 1

This Example described the various materials and procedures employed herein.

Synthesis of 20 residue peptides

For the analysis of human IgE and IgG antibody-binding epitopes, a set of 23 peptides, each 20-residue long and with 10 amino acid overlap, was designed according to the deduced amino acid sequence of the cDNA clone, KBG60 (ref. 64). The peptides were synthesized using a ABI-model 438 peptide synthesizer based on the methods described by Merrifield (ref. 65). After cleavage, the peptides were purified with reverse-phase HPLC.

Analysis of epitopes with the synthetic peptides

The binding of these peptides to the murine anti-rKBG60, and human IgE and IgG antibodies, was examined by ELISA. Briefly, each well of Nunc Maxisorb (BRL, CA) microtiter plate was coated with 10μg peptide in 100μl of 0.05M carbonateibicarbonate buffer, pH9.6, overnight at 4°C. The plates were then washed with PBS-Tween buffer three times, and the free sites of the wells were saturated with 2% BSA in PBS buffer by incubation at room temperature for 2 hours. After washing, the wells were incubated with 100μl of the murine antisera raised against the rKBG60 at 1:200 dilution. For detection of human IgG- and IgE-binding peptides, 1:50 and 1:2 diluted human sera were used, respectively. PBS containing 0.5% BSA and 0.1% Tween-20 was used as dilution buffer. The plates were incubated with the antisera at room temperature overnight, and washed three times with PBS-

Tween buffer. The bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse Ig (Zymed, CA), or goat anti-human IgG (Bio-rad, ON), or goat anti-human IgE (TAGO, CA). All the second antibodies were used at 1:2000 dilution. The colour development of the substrate was carried out at 37°C for 60 minutes and the O.D. values were read with an ELISA reader. The O.D. values were regarded as positive when the reading was more than three folds of the negative control. Each ELISA was repeated at least 3 times.

Human sera:

The serum pools from 11 grass-allergic patients and 5 non-grass allergic individuals were used as allergic and control serum, respectively, for the peptide binding studies, as described before (ref. 66).

Preparation of murine antibodies:

The capacity of each peptide to induce antibodies in vivo was examined by immunization of mice with the corresponding peptide. For this purpose, female BDF1 mice, 6 to 8 weeks old, were immunized with the synthetic peptides. Each mouse received 30μg of the peptides mixed with 2.5mg dextran sulfate in 200μl saline. Mice were boosted three times in three weeks interval, with the same amount of peptides and 1mg dextran sulfate. The blood was collected after seven days of second, third and fourth immunization. The sera were isolated and stored at -20°C and assayed at the same time. The murine anti-rKBG60 antibodies were also produced according to the method reported before (ref. 67).

Detection of the allergen-specific antibodies induced with the synthetic peptides:

The specific antibodies induced by the peptides in mice were examined by ELISA. Microtiter plates were either coated with 0.1μg per well of the rKBG8.3 protein for detection of the reactivities of the antibodies

induced with the peptides to the recombinant allergen, or coated with 10μg per well of the synthetic peptides used in immunization for detection of peptide-specific antibodies, in 100μl carbonate/bicarbonate buffer, pH9.6, overnight at 4°C. The plates were washed three times, saturated with PBS buffer containing 2% BSA and incubated with 1:20 diluted murine sera at room temperatur overnight. The bound antibodies were detected with goat anti-murine Ig conjugated with alkaline phosphatase (Zymed, CA) and the enzyme substrate, by color reaction as described above.

Preparation of PBMCs and Proliferation assay:

Venous blood (20 ml) was collected into sterile plastic syringes containing 250 u/ml of heparin, diluted 1:2 with balanced solution in 50 ml sterile centrifug tubes and underlayed with Ficoll-Hypaqe (Pharmacia) in a ratio of 1:3 according to manufacturer's instructions (Pharmacia). The tubes were centrifuged at 400 g for 30 minutes. Interphase cells were removed and washed three times, twice with balanced salt solution, and once with complete culture medium. These cells, referred to as PBMCs, were used for proliferation studies. The proliferation assays were performed in 96-well microtiter plate. The PBMCs were resuspended to a concentration of 5 X 10^6/ml in complete medium with 5% human AB^+ serum. PBMCs (5 X 10^5/well) were cultured in triplicates in the absence or presence of antigen or peptide. Four days later the culture was pulsed with [^{3}H]-thymidine for 16 hours and [^{3}H] incorporation was measured by scintillation counting. The values given are th arithmetic mean cpm of triplicate cultures.

Example 2:

This Example illustrates the binding of synthetic peptides to human IgG antibodies.

Scanning of the overlapping p ptides with the serum pool from patients allergic to KBG pollen led to the

identification of peptides #6, #9 to 12, #17, #21, #23 and #28, which were capable of binding to human IgG antibodies. These ten peptides clearly showed higher binding (more than three folds) than the background as determined by ELISA and were therefore inferred as the human IgG binding determinants (see Fig. 1, group a). The peptides #14, #18, #20 and #25 also showed IgG binding activity, although the O.D. values were not high. Assay of these peptides for their ability to bind to antibodies in the control serum pool revealed that two peptides #10 and #27 had relatively higher binding activities compared to the other peptides (see Fig. 1, group b). The N terminal region spanning the overlapping peptides from #9 to #12 appeared to contain at least one epitope. These results show that the peptides #6, #9 to 12, #17, #21, #23 and #28 encompass the epitopes of the rKBG60 involved in human IgG antibody binding.

Example 3:

This Example illustrates the human IgE-binding epitopes are localized at the C-terminus.

The synthetic peptides were also used to determine the IgE binding epitopes. The serum pool from the allergic patients recognized the peptides #12, #13, #18, #20-21, and #23-28 (see Fig. 2, group a). These peptides reacted with human IgE antibodies in repeated experiments. The control serum pool showed partial binding to some peptides, such as peptides #9, #23 and #27, and these reactions were regarded as non-specific binding (see Fig. 2, group b). The results demonstrated that IgE binding epitopes of the rKBG60 located mainly on its C-terminal region, and 10 peptides were involved in the human IgE antibody binding.

Both human IgG and IgE antibodies from the atopic serum pool recognized at least ten peptides. The peptides, #12, #21, #23 and #28 showed both IgG and IgE binding activity, whereas the peptides #18, #20 and #24

to 27 were found to be recognized exclusively by the human IgE antibody from the sera. The peptides #6, #9 to 11, and #17, showed no binding activities to human IgE antibodies. Taken together, these results indicated that most but not all human IgG and IgE antibodies from the same serum pool recognized the same peptides of the rKBG60.

Example 4:

This Example illustrates that murine anti-rKBG60 sera recognize synthetic peptides.

For comparison of epitopes recognized by human IgE and IgG antibodies with the epitopes defined the murine antibodies, the synthetic peptides were also analyzed for their reactivity with murine anti-rKBG60 antisera. The results presented in Fig. 3, group a indicate that peptides #7, #12 to 19, #21, #23, and #26 to 28 bound to anti-rKBG60 antibodies, whereas the control sera showed no detectable reactivity (see Fig. 3, group b). Thes results indicate that the peptides #7, #12 to 19, #21, #23 and #26 to 28 contain epitopes recognized by murine B cells.

Example 5:

This Example illustrates stimulation of human T-cells by several synthetic peptides.

The T-cell epitopes of rKBG60 were examined by determining the capacity of synthetic peptides to directly stimulate PBMCs from grass pollen allergic patients. Initially, using peptides #13 and #26 a dose response curve was established; the maximal proliferative response was obtained at a peptide concentration of 100μg/ml (data not shown). In total, PBMC proliferation was examined in 13 patients using a number of overlapping peptides; the data for 8 patients with selected peptides are illustrated in Figure 4 and peptides exhibiting a stimulation index (SI = 3) are shown in Table 2. Results indicated that the PBMC of different individuals had

varied levels of proliferative response to different peptides. For each individual, the mean proliferative responses with at least three peptides showing the minimal cpm values was considered background. Except for three individuals, the rest of the group responded to peptide #13, while eight of nine subjects showed response to peptides #25 and #26. Hence, peptides 13, 25 and 26 include immunodominant epitopes. Some individuals also responded to peptides #18, #23, #27 or #28. These results show that the allergen consists of several potential T-cell epitopes.

Example 6:

This Example illustrates induction of antibodies that recognize rKBG60 by synthetic peptides.

To examine the possibility that T-cell and B-cell epitopes may coincide on the same peptide, nine of these peptides, i.e., peptides #14, #16, #18, #19, #21, #23, #26, #27, and #28, which bound to the murine antibodies, and three peptides, i.e., #5, #22, and #25, which did not bind to the murine antibodies, were used to induce the antibody production in mice. Peptide-specific antibodi s were detected after tertiary immunization of the mice. The peptides which showed no antibody induction after tertiary immunization failed to induce antibody after further immunization. Among the peptides examined, eight induced the allergen-specific antibodies, whereas four peptides including the two non-antibody binding peptides, #5 and #22, and two antibody-binding peptides, #14 and #21, induced no detectable antibodies (see Fig. 5). In contrast, peptide #25, which neither bound to murine Ab nor to human IgG but bound to human IgE, induced high titers of peptide-specific antibodies. From the reactivities of the peptide-induced antisera, it is inferred that peptides #16, #18, #19, #23, #25, #26, #27 and #28 were immunogenic. Non-immune and pre-immune sera were used as controls, which showed no reactivity to the

allergen. It is concluded that eight of twelve peptid s examined were capable of inducing specific antibodies, all of which recognized the allergen rKBG8.3.

Example 7:

This Example illustrates detection of an immunodominant conformational epitope on rKBG60.

The binding abilities of the anti-peptide sera to itself with other five peptides as negative controls, are illustrated in Fig. 6. The first set consisted of peptides #5, #18, #19, #21, #22, and #23 (Fig. 6, group a). The sera raised against peptides #19 and #23 were found to react with the corresponding peptides, whereas the sera against peptides #5 and #22 did not. Furthermore, the sera against peptides #18 and #22 bound to the adjacent peptides #19 and #23, respectively; but not to the peptide which was used as immunogen or to other peptides. The sera against peptides #16, #25, #26, and #27, showed binding to the corresponding peptide used as immunogen as well as to the other coated peptides except the antisera against peptides #14 and #28 (see Fig. 6, group b). Similar to anti-peptide #18 serum, anti-peptide #28 showed binding to the adjacent peptides #25 to #27 but not to itself. The antisera against peptides #25 to #28 appeared to share some common specificities.

These results show that the peptides #25 to #28 include an immunodominant epitope.

Example 8:

This Example shows that different allergen peptides may induce different T-cell responses.

The mice were immunized and subsequently boasted with the peptides and antigen rKBG8.3 as described in Example 1. the IgG_{2a} and IgE antibody isotypes were examined specifically representing Th1 and Th2 type of responses, respectively. The data obtained (Table 3 below) demonstrate that peptide #26 is capable of

inducing a high IgG_{2a} response in mice and suggest this should induce a protective response in allergic individuals.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides certain novel synthetic peptides, which may be made by chemical synthesis or by recombinant techniqu s, which contain human T-cell and/or B-cell epitopes of Kentucky Blue grass allergens from the Poa p IX group of grass pollen allergens, particularly the KBG allergen rKBG60. Modifications are possible within the scope of this invention.

TABLE 1

Amino acid sequences of the 20-residue peptides

Peptide Number	Positions on the rKBG60	Amino Acid Sequence	SEQ ID NOS:
6	29-48	APKATTDEQKLIEKINVGFK	7
7	39-58	LIEKINVGFKAAVAAAGGVP	8
9	59-78	AANKYKTFVATFGAASNKAF	9
10	69-88	TFGAASNKAFAEALSTEPKG	10
11	79-98	AEALSTEPKGAAVDSSNAAL	11
12	89-108	AAVDSSNAALTSKLDAAYKL	12
13	99-118	TSKLDAAYKLAYKSAEGATP	13
14	109-128	AYKSAEGATPEAKYDDYVAT	14
16	129-148	LSEALRIIAGTLEVHGVKPA	15
17	139-158	TLEVHGVKPAAEEVKATPAG	16
18	149-168	AEEVKATPAGELQVIDKVDA	17
19	159-178	ELQVIDKVDAAFKVAATAAN	18
20	169-188	AFKVAATAANAAPANDKFTV	19
21	179-198	AAPANDKFTVFEAAFNDAIK	20
23	199-218	ASTGGAYQSYKFIPALEAAV	21
24	209-228	KFIPALEAAVKQSYAATVAT	22
25	219-238	KQSYAATVATAPAVKYTVFE	23
26	229-248	APAVKYTVFETALKKAITAM	24
27	239-258	TALKKAITAMSQAQKAAKPA	25
28	249-268	SQAQKAAKPAAAATGTATSA	26

TABLE 2

Human B- and T-Cell Recognition of Synthetic Peptide of rKBG60 Allergen

Peptide Number	B-Cell Responses hIgG	hIgE	T-Cell Responses
6	+	-	+
7	-	-	-
9	+	-	+
10	+	-	+
11	+	-	-
12	+	+	-
13	-	+	+
14	-	-	+
16	-	-	-
17	+	-	+
18	-	+	+
19	-	-	+
20	-	+	+
21	+	+	+
23	+	+	+
24	-	+	-
25	-	+	+
26	-	+	+
27	-	+	+
28	+	+	+

TABLE 3

Allergen Specific IgG_{2a} and IgE Titers of Mice Immunized with Poa p IX Peptides

Peptides	Days after immunization					
	Day 8		Day 15		Day 29	
	IgG_{2a}	IgE	IgG_{2a}	IgE	IgG_{2a}	IgE
Peptide #23	ND	ND	160	ND	5120	640
Peptide #26	80	ND	160	20	40960	640
Control	20	ND	80	80	5720	1280

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CLAIMS

What we claim is:

1. A synthetic peptide having an amino acid sequence which includes at least one human antigenic determinant of a Kentucky Blue grass (KBG) allergen from the Poa p IX group of grass pollen allergens.

2. The synthetic peptide of claim 1 wherein said KBG allergen is KBG 60.

3. The synthetic peptide of claim 1 wherein said human antigenic determinant comprises a human T-cell epitope.

4. The synthetic peptide of claim 1 wherein said human antigenic determinant comprises a human B-cell epitope.

5. The synthetic peptide of claim 1 wherein said synthetic peptide has an amino acid sequence corresponding to one of the sequences shown in Table 1 which exhibits a human B-cell and/or a human T-cell response.

6. The synthetic peptide of claim 5 wherein said peptide is selected from the group consisting of peptides #s 6, 9, 10, 11, 12, 17, 21, 23 and 28 in Table 1.

7. The synthetic peptide of claim 5 wherein said peptide is selected from the group consisting of peptides #s 12, 13, 18, 20, 21, 23, 24, 25, 26, 27 and 28 in Table 1.

8. The synthetic peptide of claim 5 wherein said peptide is selected from the group consisting of peptides #s 6, 9, 10, 13, 14, 17, 18, 19, 20, 21, 23, 25, 26, 27 and 28.

9. The synthetic peptide of claim 5 which is peptide 13 or 14 in Table 1.

10. The synthetic peptide of claim 5 which is peptide 13, 25 or 26 in Table 1.

11. A composition for protecting an allergic individual from developing an allergic reaction to grass pollen, comprising at least one synthetic peptide as claimed in

claim 1 and a pharmaceutically-acceptable carrier therefor.

12. The composition of claim 11 formulated as a vaccine for *in vivo* administration.

13. The composition of claim 12 wherein said vaccine comprises said at least one synthetic peptide conjugated to a non-immunogenic substrate.

14. The composition of claim 13 wherein said non-immunogenic substrate is a polymeric material.

15. The composition of claim 14 wherein said polymeric material is selected from the group consisting of carboxymethyl celluloses, monomethoxypolyethylene glycols and polyvinyl alcohols.

16. The composition of claim 13 wherein said non-immunogenic substrate comprises beads for targeted uptake of said at least one recombinant protein by selected antigen-presenting cells.

17. The composition of claim 16 formulated as a microparticle, capsule or liposome preparation.

18. The composition of claim 11 in combination with a targeting molecule for delivery to specific cells of the immune system or to mucosal surfaces.

19. The composition of claim 12 comprising at least one additional desensitizing agent.

20. The composition of claim 19 wherein said at least one additional desensitizing agent is selected from the group consisting of *Lol p* I allergen, *Bet v* I allergen, *Amb a* I allergen, *Amb a* II antigen and CRAL 51 allergen.

21. The composition of claim 12 further comprising at least one compound having anti-histamine activity and/or at least one compound have anti-inflammatory activity and/or at least one compound which is immunosuppressive.

22. The composition of claim 12 further comprising an adjuvant.

23. A method for desensitizing an allergic individual, comprising administering to the individual an effective amount of the composition of claim 11.

24. The method of claim 23 wherein the individual is a human.

25. The method of claim 24 wherein said immunogenic composition is that of claim 20.

26. A method of depleting allergen-specific antibodies from an individual, comprising contacting said antibodies with said composition of claim 11 to form a complex, and removing the complex from the individual.

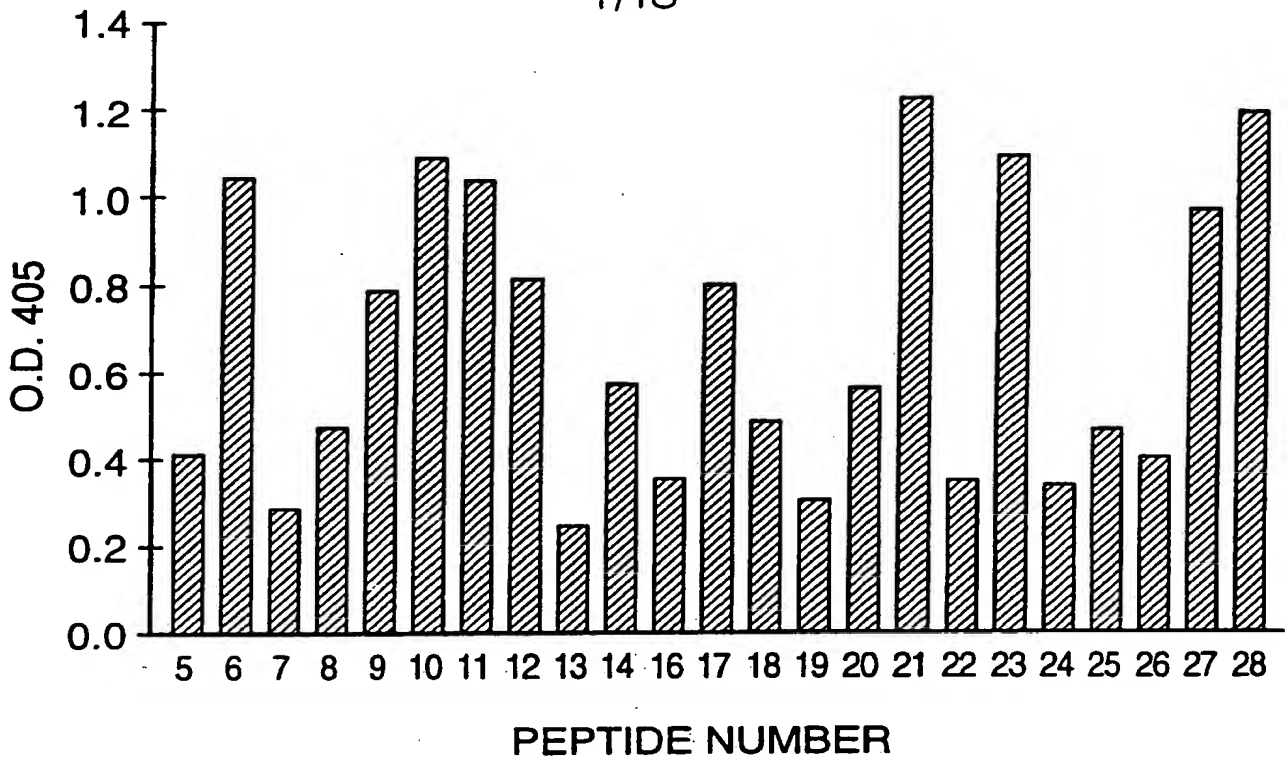
27. The method of claim 26, wherein said individual is a human.

28. A method of anergizing allergen-specific antibody-producing cells, comprising contacting said cells with said composition of claim 13.

29. An antiserum specific for a synthetic peptide as claimed in claim 1.

30. A method for diagnosing an allergic reaction to grass pollens, which comprises administering to an individual with a synthetic peptide of claim 1, and evaluating a response to said administration.

31. A method for diagnosing an allergic reaction to grass pollen, which comprises contacting serum from an individual with a synthetic peptide of claim 1, and determining the formulation of a complex between the synthetic peptide and pollen-specific IgE antibodies present in the serum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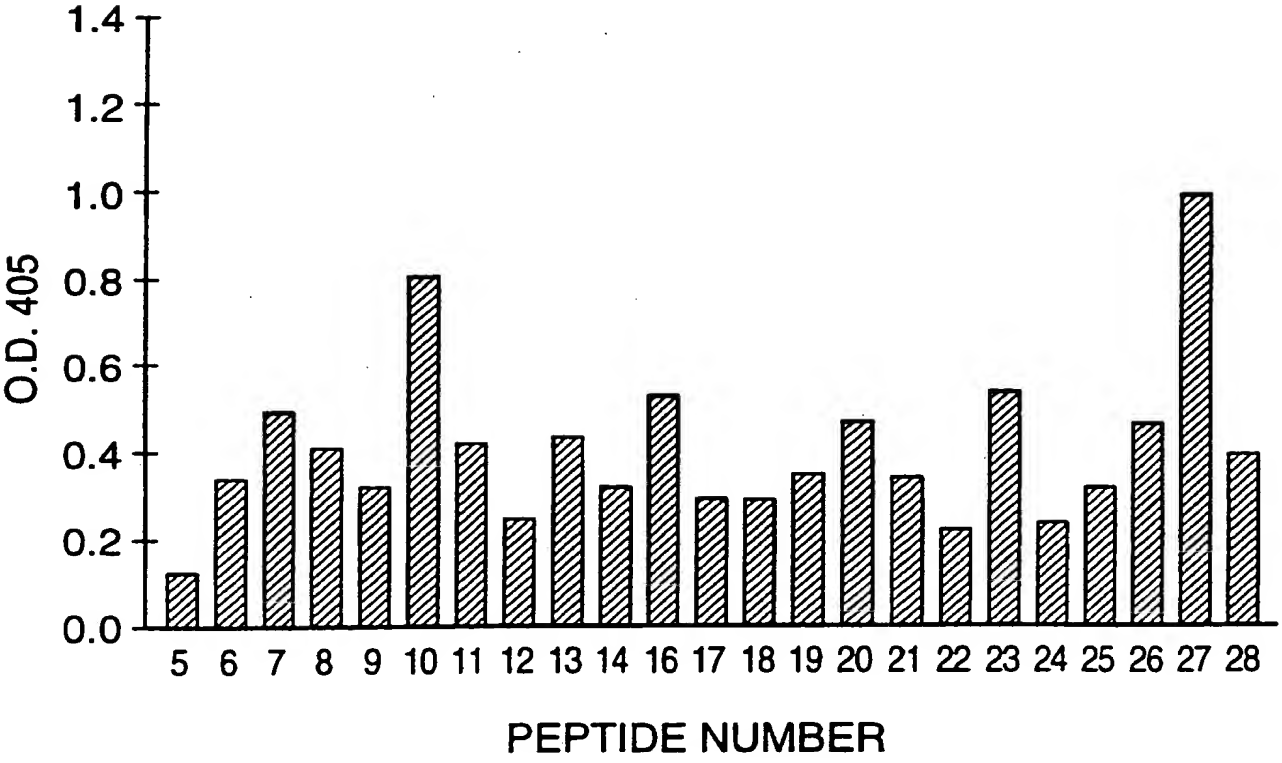
PEPTIDE NUMBER

FIG.1A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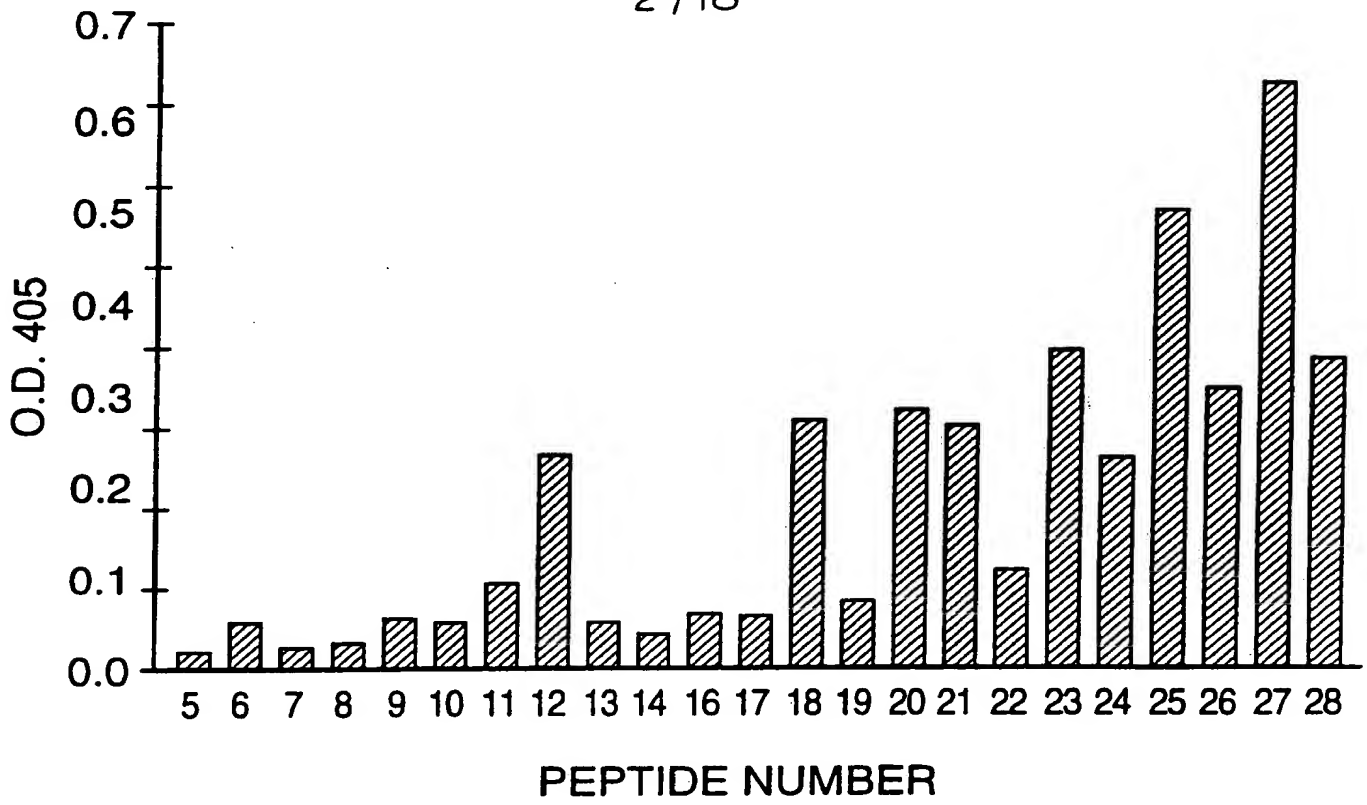
PEPTIDE NUMBER

FIG.1B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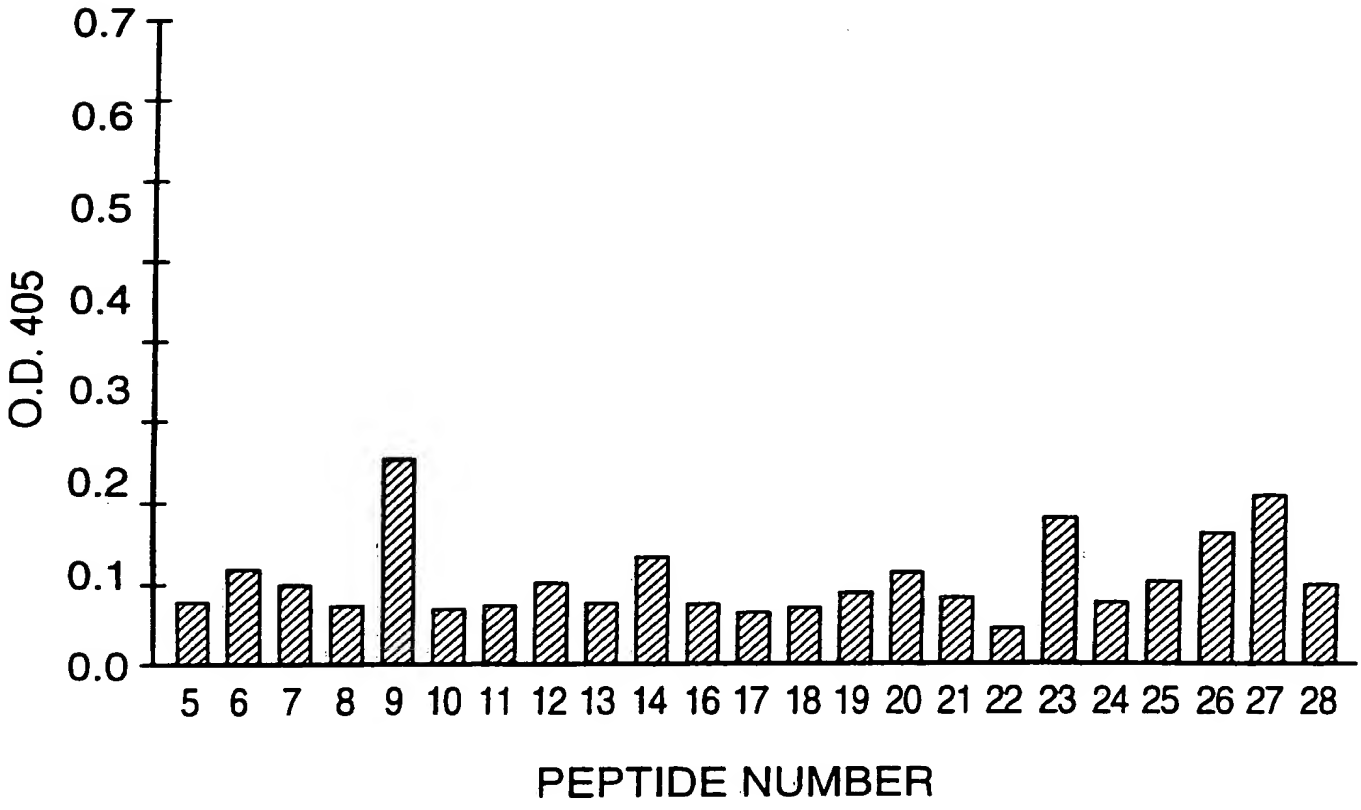
PEPTIDE NUMBER

FIG.2A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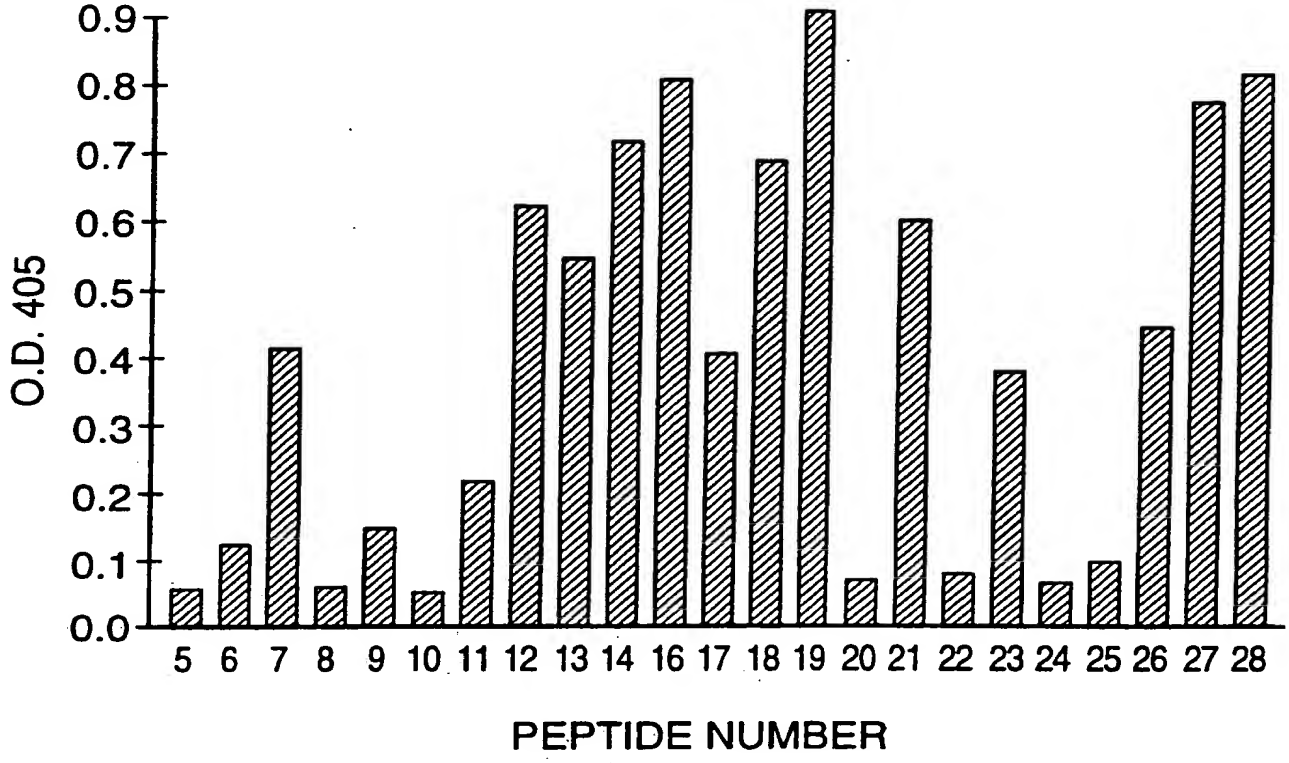
PEPTIDE NUMBER

FIG.2B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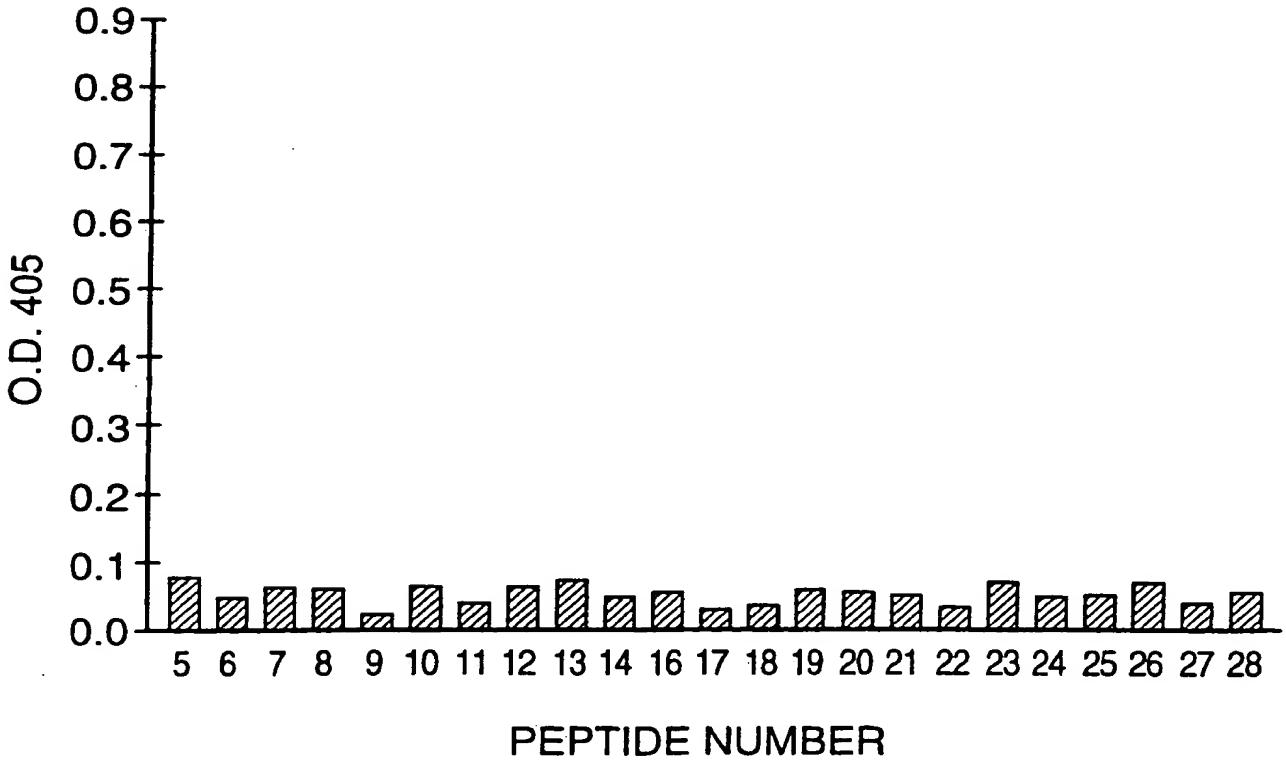
PEPTIDE NUMBER

FIG.3A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0.3
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O.D. 405
5 6 7 8 9 10 11 12 13 14 16 17 18 19 20 21 22 23 24 25 26 27 28
PEPTIDE NUMBER

FIG.3B

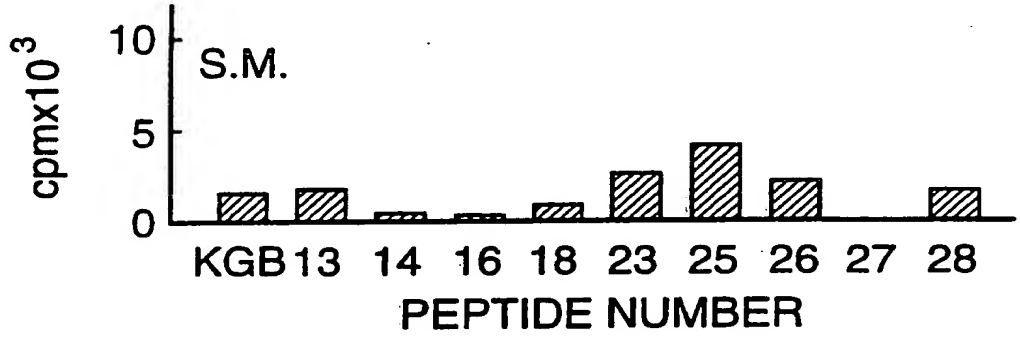


FIG.4A

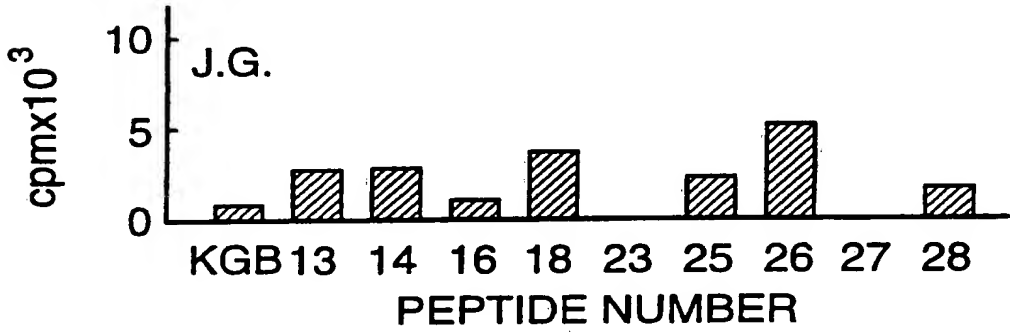


FIG.4B

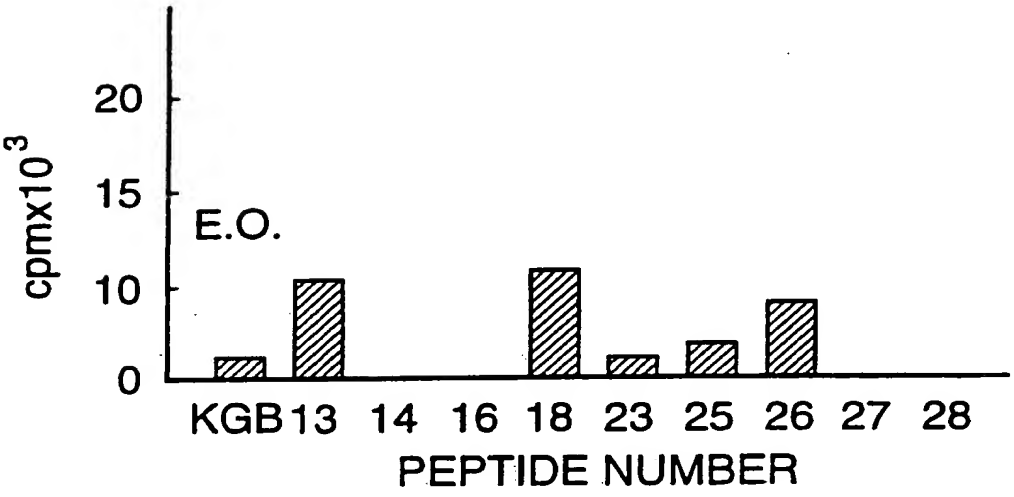


FIG.4C

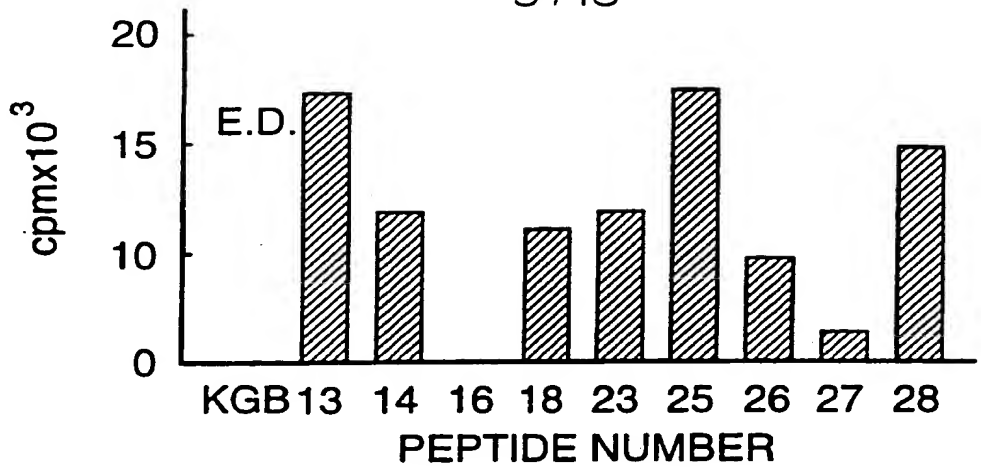


FIG.4D

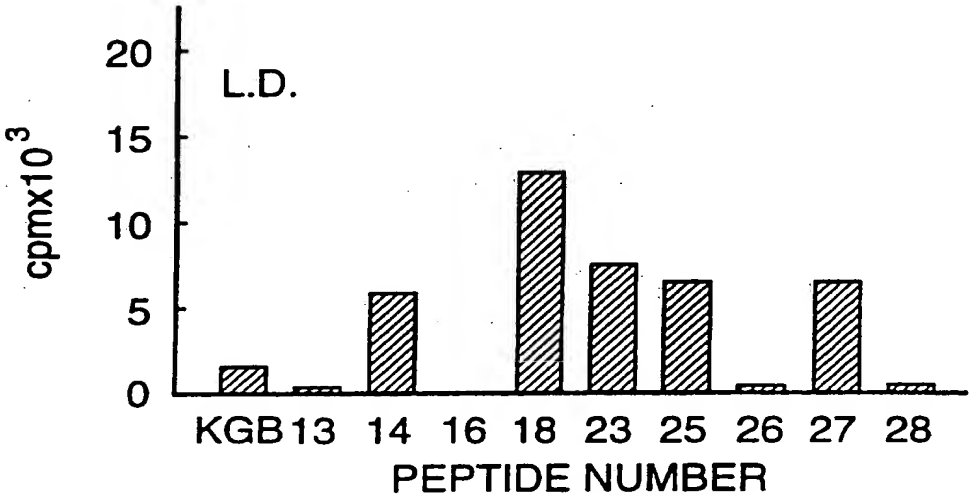


FIG.4E

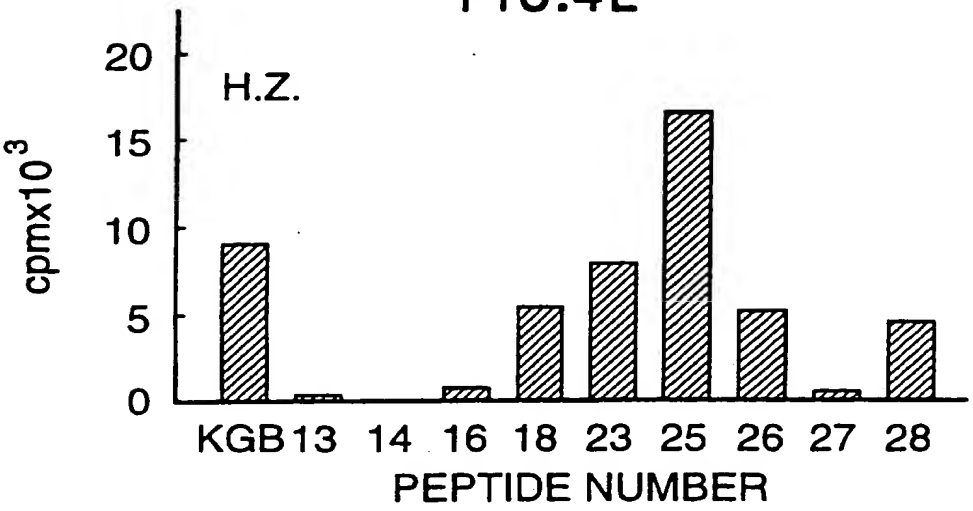
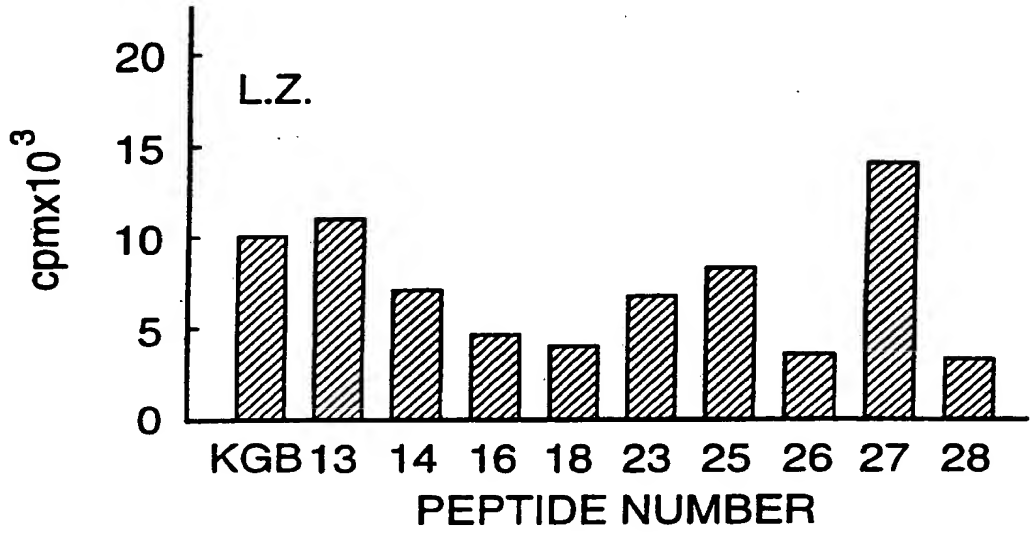


FIG.4F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10
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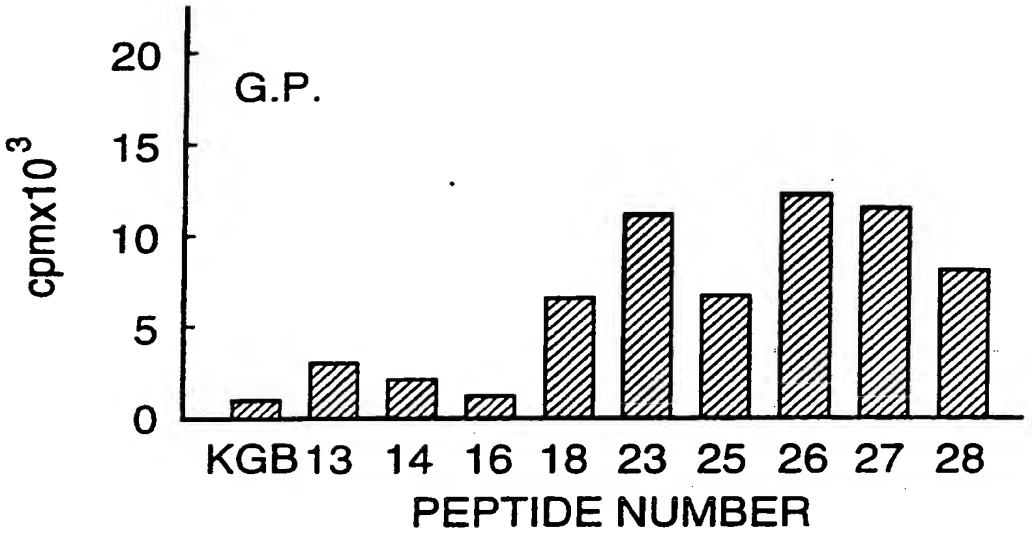
0
cpmx10^3
L.Z.
KGB 13 14 16 18 23 25 26 27 28
PEPTIDE NUMBER

FIG.4G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10
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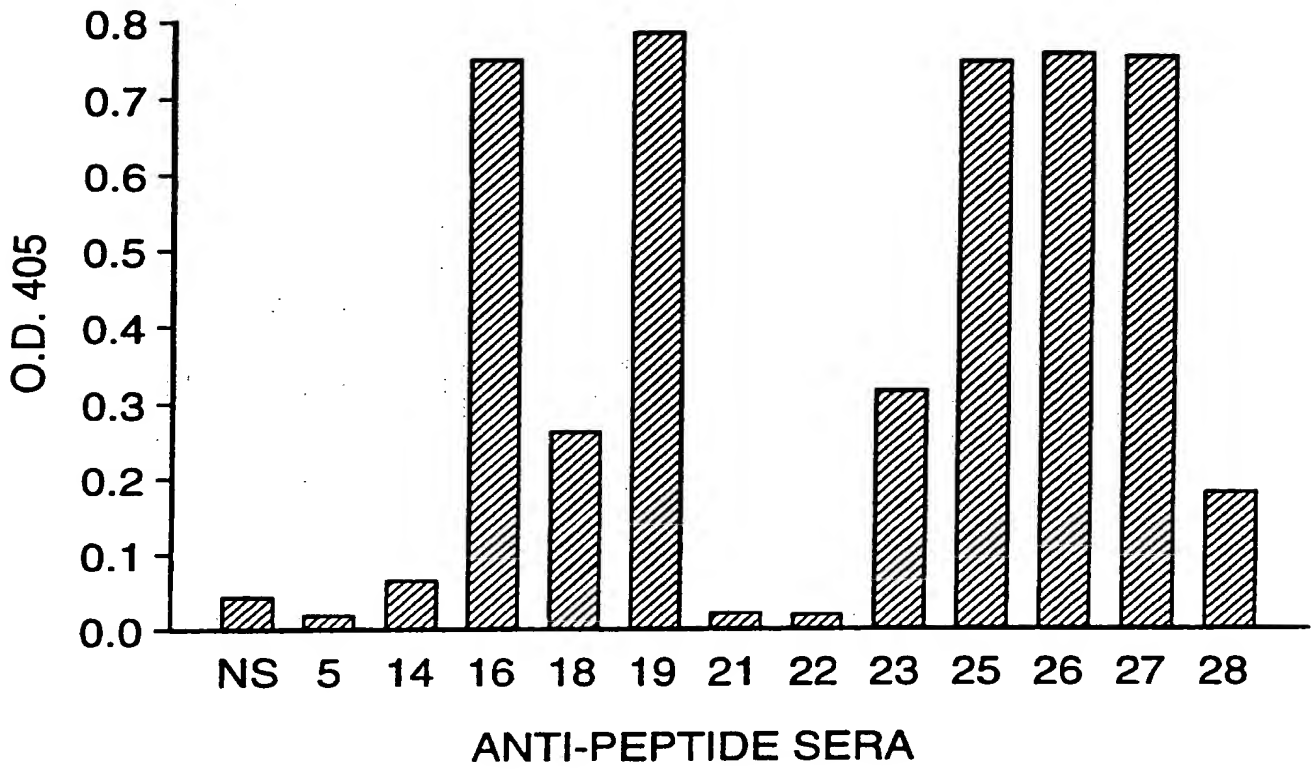
0
cpmx10^3
G.P.
KGB 13 14 16 18 23 25 26 27 28
PEPTIDE NUMBER

FIG.4H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O.D. 405
NS 5 14 16 18 19 21 22 23 25 26 27 28
ANTI-PEPTIDE SERA

FIG.5

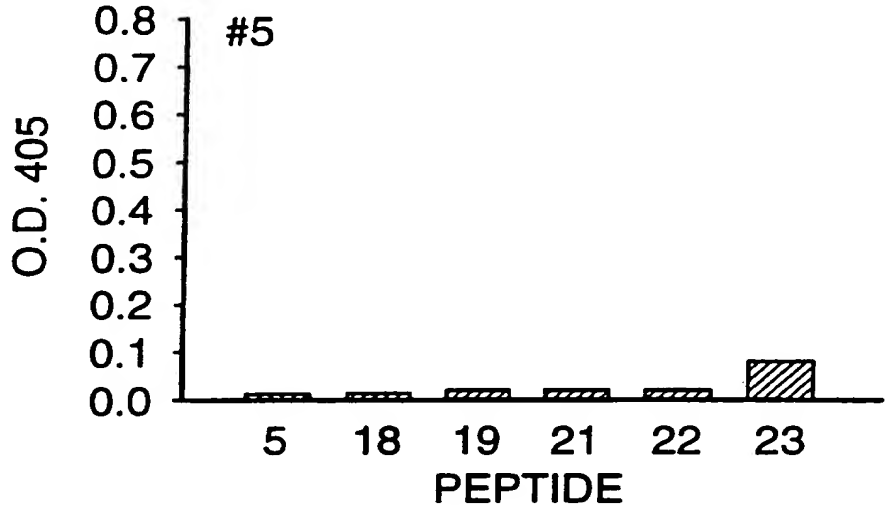

FIG.6A

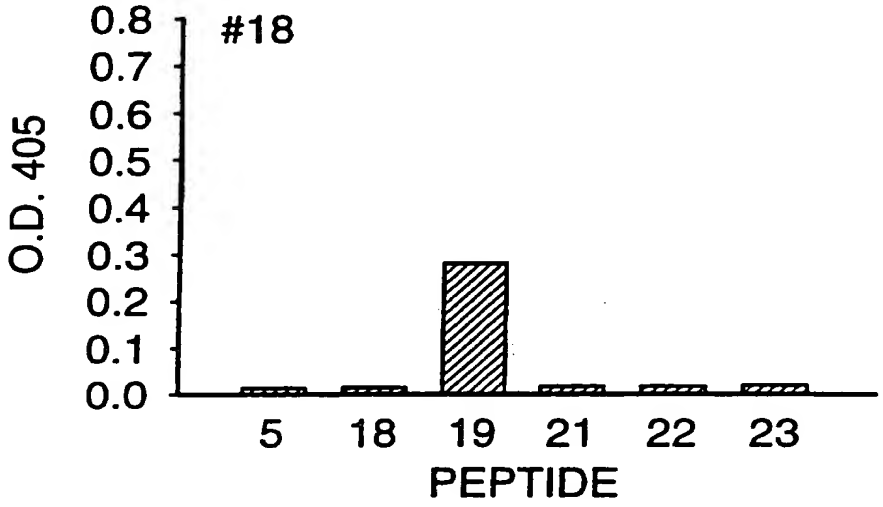

FIG.6B

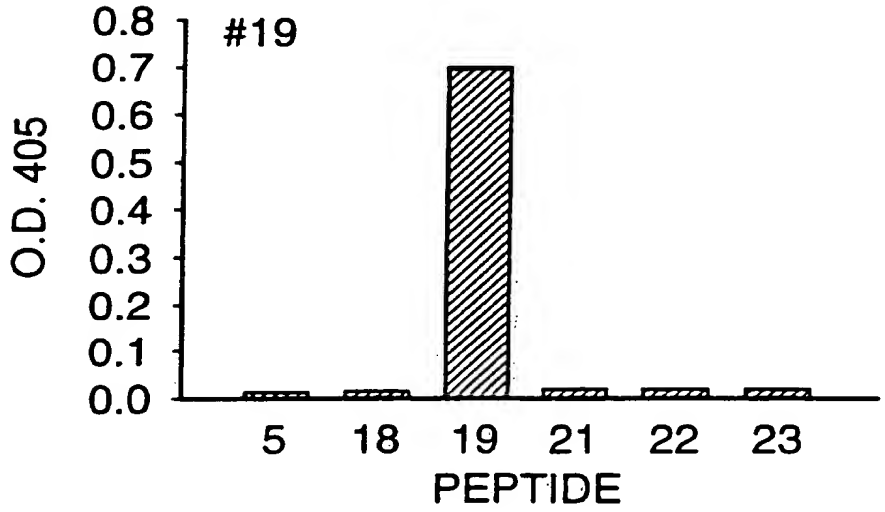

FIG.6C

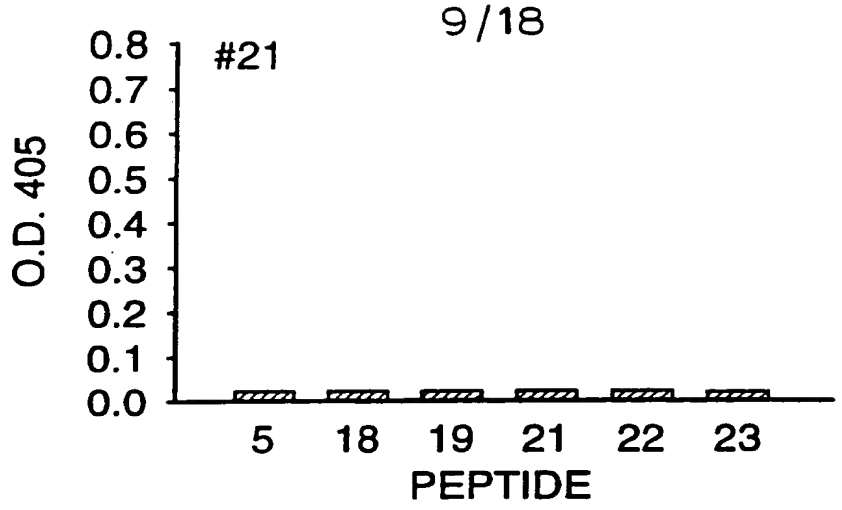

FIG.6D

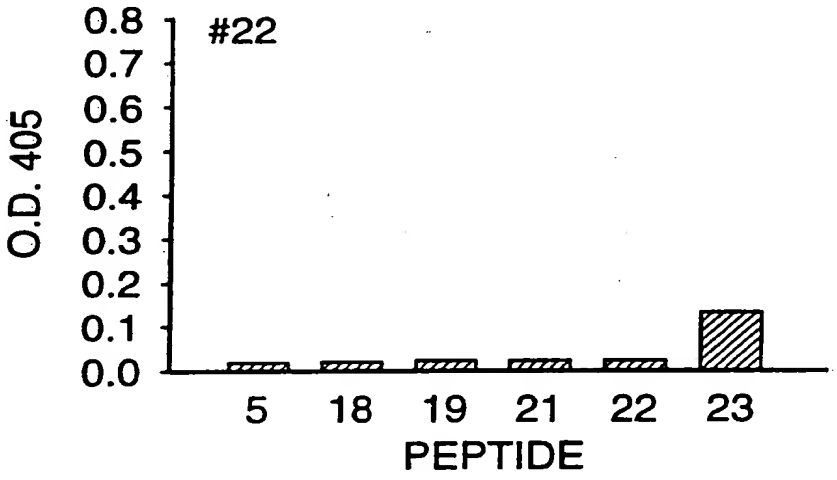

FIG.6E

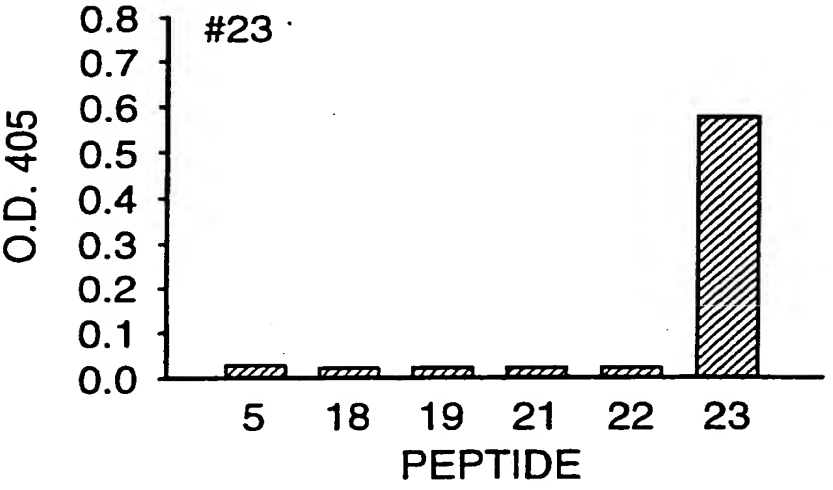

FIG.6F

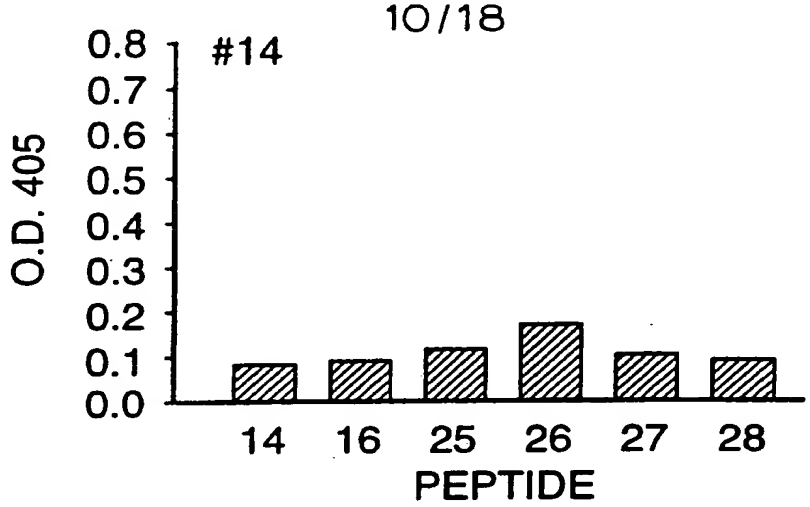

FIG.6G

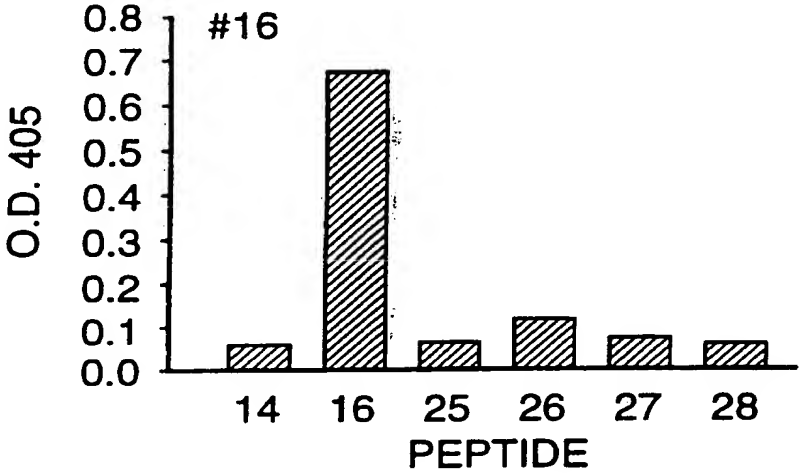

FIG.6H

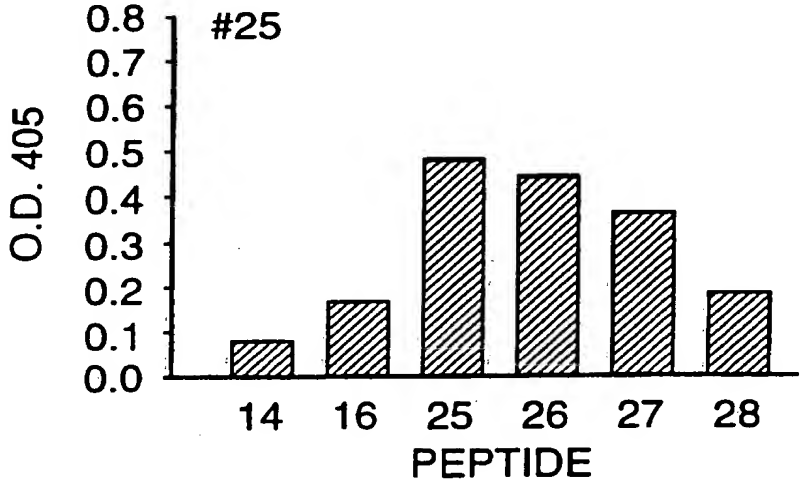

FIG.6I

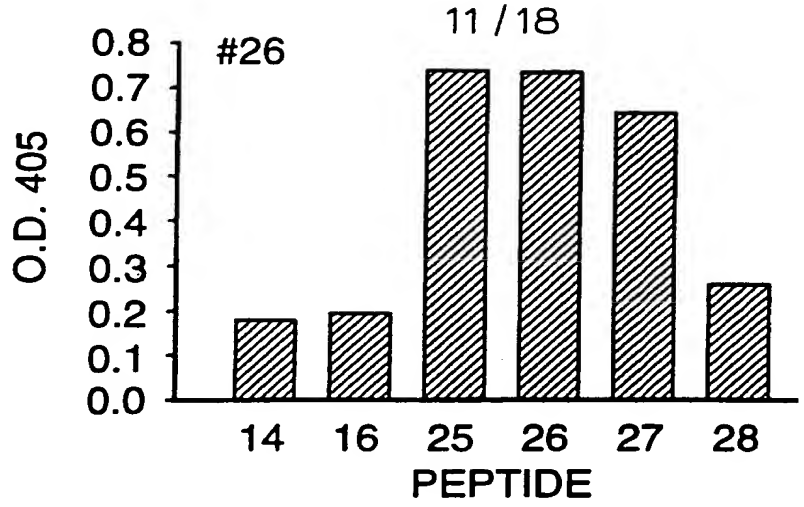

FIG.6J

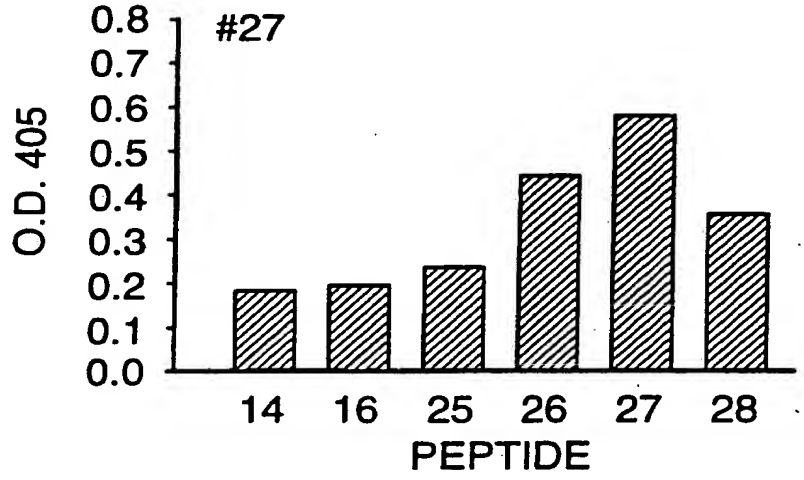

FIG.6K

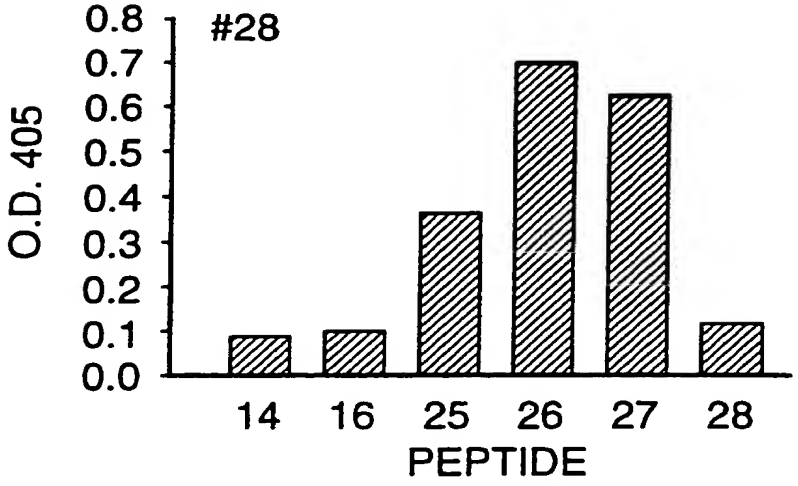

FIG.6L

FIG.7A.

```
41  -142
60                                                                  gaattc
31
41    -136    TTTTTTTTTTTTTGATTTGAAGCGAAAATATATCCCATCACCACCAAATAAAAATGTTACAGTTGATC
60    -85
31     -6                                                       gaattcnnnnnATGAT
                                                                    gaattc

41      -69  CCTAACACAAATCACAACACAATCCATCCCATATCTTCCCACCTACGGTACAAACAACAGCAAGCAGGA
60      -69  GAAACGAAAACCGAAGAATTAATCCATCGCATATCTTTCCTCCTGCGATACAAACCAACAACAGCAGTA
31

41       ] ATG GCG GTG CAC CAG TAC ACT GTG GCG CTG TTC CTC GCC GTG GCC CTC GTG
60       ] ... ..G ... ..G A.. ... ..G ..C ... ... ... ..G     ... ... ... ...
31       ] ... .AC AA. GC. A.C GGG G.G TAC AA. AC. GC. ..G AAG .C. ... AG. .C.

41      52 GCG GGC CCG GCT GCC TCC
60      49 .TT ... ... ... ... ...
31      52 .T. .C. ... ..G .AG AAG TTC CCG GTG TTC CAG GCC ACC TTC GAC AAG AAC

41
60
31    103 CTC AAG GAG GGC TTG TCC GGT CCC GAC GCC GTC GGG TTC GCC AAG AAG CTC

41
60
31    154 GAC GCC TTC ATC CAG ACC TCC TAC CTC AGC ACC AAG GCC GCC GAG CCC AAG
```

FIG.7B.

41	70														TAT	GCT	GCC	
60	67														...	..C	..T	
31	205	GAG	AAG	TTC	GAC	TTA	TTC	GTG	CTC	TCA	CTG	ACG	GAG	GTG	CTC	CG.	TT.	ATG
41	79	GAC	GTC	GGC	TAC	GGC	GCC	CCC	GCC	ACC	CTT	GCC	AGC	CCT	GCC	ACC	CCT	GCT
60	76	...	...	A..	...	...	...	...	...	.A.	.C.							...
33	256	.CG	.G.	.C.	GTG	AAG	...	..G	C..	G..	AGC	AAG	TT.	..C	...	.AA	..C	...
41	124	GCC	CCG	GCG	GCC	GGC	TAC	ACC	CCC	GCC	GCC	CCG	GCC	GGA	GCG	GCA	CCA	AAG
60	103	...	...	..C	...	.CT	...	...	...	...	...	...	...	...	...	...	...	...
31	307	C..	AA.	.T.	...	.CT	...	...	...	...	...	...	...	...	...	...	...	...
41	181	GCG	ACG	ACC	GAT	GAG	CAG	AAG	CTG	ATC	GAG	AAG	ATC	AAC	GCC	GGC	TTC	AAA
60	160	...	...	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	...
31	358	...	..C	...	...	...	...	...	...	...	...	...	...	...	.T.	...	...	..G
41	232	GCG	GCC	GTT	GCC	GCC	GCC	GCC	GGC	GTC	CCA	GCA	GTG	GAC	AAG	TAC	AAG	ACG
60	211	...	...	...	...	...	...	.G.	...	...	..G	...	.CC	A..	...	...	...	...
31	409	..T	...	...	...	...	..A	...	...	...	..G	...	.C.	AG.	...	...	...	...
41	283	TTC	GTC	GCA	ACC	TTC	GGC	ACG	GCC	TCC	AAC	AAG	GCC	TTT	GCG	GAG	GCC	CTC
60	262	...	...	...	..G	...	...	G..	...	...	...	...	...	...	...	...	...	..A
31	460	...	..A	...	...	...	...	G..	...	...	...	...	...	..C	..C	...	...	...

FIG.7C.

41	334	TCC	ACC	GAG	CCC	AAG	GGC	GCC	GCC	GCA	GCC	AGC	TCC	AAT	GCT	GTT	CTC	ACC
60	313	..G	...	...	...	...	...	...	...	.T.	.A.	...	...	..G	..C	.CG	...	...
31	511	..G	...	...	...	...	...	...	...	.T.	...	...	...	..G	..C	..G	...	...
41	385	TCC	AAG	CTC	GAC	GCC	GCC	TAC	AAG	CTC	GCG	TAC	AAG	TCA	GCG	GAG	GGC	GCG
60	364	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...
31	562	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...
41	436	ACG	CCC	GAG	GCT	AAG	TAC	GAC	GCC	TAC	GTC	GCC	ACC	CTA	AGC	GAG	GCG	CTC
60	415	...	...	...	...	...	...	...	.A.	...	...	...	...	..T	...	...	...	...
31	613	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
41	487	CGC	ATC	ATC	GCC	GGC	ACC	CTC	GAG	GTC	CAC	GCC	GTC	AAG	CCC	GCG	GGC	GAG
60	466	...	...	..T	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...
31	664	...	...	...	...	...	...	...	...	...	...	.G.	...	...	...	...	...	...
41	538	GAG	GTC	AAG	GCC	ATC	CCC	GCC	GGC	GAG	CTG	CAG	GTC	ATC	GAC	AAG	GTC	GAC
60	517	...	...	...	...	.C.	...	...	...	...	..C	...	...	...	...	...	...	...
31	715	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
41	589	GCC	GCC	TTC	AAG	GTC	GCT	GCC	ACC	GCC	GCC	AAC	GCC	GCC	CCC	GCC	AAC	GAC
60	568	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
31	766	...	...	...	...	...	...	...	...	...	..G	...	...	...	...	...	...	...

FIG.7D.

```
41    640 AAG TTC ACC GTC TTC GAG GCC GCC TTC AAC GAT GCC ATC AAG GCG AGC ACG
60    619 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
31    817 ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ... ... ...

41    691 GGC GGC GCT TAC CAG AGC TAC AAG TTC ATC CCC GCC CTC GAG GCC GCC GTT
60    670 ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ..C
31    868 ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ..C

41    742 AAG CAG TCC TAC GCC GCC ACC GTC GCC ACC GCG CCG GCG GTC AAG TAC ACC
60    721 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
31    919 ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ..T

41    793 GTC TTT GAG ACC GCG CTG AAA AAG GCC ATC ACC GCC ATG TCC CAG GCC CAG
60    772 ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
31    970 ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ...

41    844 AAG GCT GCT AAG CCC GCC GCT GCT GTC ACC GCC ACC GCA ACC GGG GCG GTT
60    823 ... ... ..C ... ... ... ... ... .C. ... ... ... ... ... .CT ..C ..C
31   1021 ... ... ... ... ... ..T ..C ... ... ... ... ... ..G ... A.C ..C ..C

41    895 GGC GCG GCC ACC GGC GCC GTC GGC GCG GCA ACC GGC GCT GCC ACC GCC GCT
60    874 ... ... ..A ...
31   1072 ... ... ..A ...
```

FIG.7E.

```
41    946 GCT GGT GGC TAC AAA ACC GGC GCT GCC ACC CCC ACT GCT GGT GGC TAC AAA
60    886                                 ... ... ... ... G.. G.. ... ... ... ... ...
31   1084                                 ... ... ... ... G.. G.. ... ... ... ... ...

41     997  GTC  TGA  TCTGCTCCCAATATACACCCATCGTGCATATGTGTACAACTGTATGTATGTGCATGCATGG
60     919  ...  ...  TCTGCTCCCTACAATATACATCCATCATGCACATGTACAACTGTATGTATGTGCATGCATG
31    1117  ...  ...  TCTGCTCCCTAACAATATACATCCATCATGATTCATGCATGGCCGCCGCGTCGTGCGATTT

41    1064  CCGGCCGCCGGGTCGTGCGCTTTTGCTCATAATTAATTTTTGGGTTTTCGTTTCATGCATGCATCCGCG
60     986  GCCGCCGCCTCGTGCGATTTTGCTCATAATTAATTCTTCGGTTTTCGATTCATGCATCCGCGAGCTATA
31    1184  TGCTCATAATTAATTTTTGGGCGTTCGTTTCATCGATCGCAAGCTATAAAGCGCATGCATGCATGATCC

41    1133  AGCTATAGAGCGCATGCATGCATGATCATTTGTGTAATATAATAATTAAATATTTGTAATTCATTTACA
60    1053  GAGCGCATGCGCATGATCATTTGTGTAATATAATAATTAAATATTTGTAATTTATTTACAAGTATGACG
31    1253  TTTGTTTAATATCATAATTAAATATTTGTAATTTATTTACAAGTAAAAAAAAAAAAAGgaattc

41    1201  AGTAAAAAAAAAAGgaattc
60    1121  AAAAAAAgaattc
```

cDNA sequences of KBG clones 41, 60, and 31.

FIG.8A.

```
41   -23 mavhqytvalflavalvagpaas
60   -22 ...qk.......v. ..v.....
31   -28 .dkangaykta.ka.sava..ekfpvfqATFDKNLKEGLSGPDAVGFAKKLDAFIQTSYL

41     1                              YAADVGYGAPATLATPATPAAPAAGYTPAAPAGAAPKA
60     1                              ....LS.....      ......A.........____
31    33 STKAAEPKEKFDLFVLSLTEVLRFMAGAVK..PASKF..K..PKV.A.........______

41    39 TTDEQKLIEKINAGFKAAVAAAAGVPAVDKYKTFVATFGTASNKAFAEALSTEPKGAAAA
60    33 ............V.........G....AN.........A..................VD
31    93 ____________.V.............AS.........A.......__________..V.

41    99 SSNAVLTSKLDAAYKLAYKSAEGATPEAKYDAYVATLSEALRIIAGTLEVHAVKPAGEEV
60    93 ....A.........................D..................G....A...
31   153 ..................____________.....................G____A___

41   159 KAIPAGELQVIDKVDAAFKVAATAANAAPANDKFTVFEAAFNDAIKASTGGAYQSYKFIP
60   153 ..T.....................................................
31   213 _.....................................__________.........
```

FIG.8B.

```
41   219 ALEAAVKQSYAATVATAPAVKYTVFETALKKAITAMSQAQKAAKPAAAVTATATSAVGAA
60   213 ....................................................A.G.........
31   273 ..............................................................G.........

41   279 TGAVGAATGAATAAAGGYKTGAATPTAGGYKV
60   254 .                    .....AA......
31   333 .                    .....AA......
```

Translated sequences of KBG clones 41, 60, and 31.